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(54) Title: EARLY DETECTION OF TRANSGENIC EMBRYOS

(57) Abstract

The present invention provides methods, based on in *situ* hybridization and the polymerase chain reaction, for the early detection of integrated transgenes in the nuclear genome.

EARLY DETECTION OF TRANSGENIC EMBRYOS

5

BACKGROUND OF THE INVENTION

In recent years there have been numerous reports of the production of transgenic animals, most of which relate to the production of transgenic mice. See, inter alia, U.S. Pat.

10   No. 4,736,866; Andres et al., Proc. Natl. Acad. Sci. USA 84:1299-1303 (1987); Schoenberger et al., Experientia 43:644 (1987), and EMBO J. 7:169-175 (1988); and Muller et al., Cell 54:105-115 (1988), all incorporated herein by reference. Several laboratories have also reported the production of

15   transgenic porcine species (Miller et al., J. Endocrin. 120:481-488 (1989); Vize, P.D., et al., J. Cell Sci. 90:295-300 (1988); and Ebert et al., Mol. Endocrin. 2:277-283 (1988); all incorporated herein by reference), transgenic sheep (Nancarrow et al., Theriogenology 27:263 (1987); Clark et al.,

20   Bio/Technology 7:477-482 (1989) and Simons et al., Bio/Technology 6:179-183 (1988); all incorporated herein by reference), and rabbit (Hanover et al., Deutsche Tierarztliche Wochenschrift 94:476-478 (1987), incorporated herein by reference).

25   A number of reports have also suggested the production of transgenic cattle (e.g., Wagner et al., Theriogenology 21:29-44 (1984); Logse et al., Theriogenology 23:205 (1985); all incorporated herein by reference). A recent article has summarized the genetic engineering of livestock.

30   (Pursel et al. Science 244:1281-1288 (1989); incorporated herein by reference). Methods for production of recombinant polypeptides in the milk of bovine species and methods for producing transgenic animals having desired phenotypes are discussed in co-pending U.S.S.N. 444,745 and 619,131;

35   incorporated herein by reference.

High costs and long gestation periods of large animals make it difficult, time consuming and expensive to produce transgenic livestock. Current techniques to produce

transgenic animals involve transfer of all microinjected
embryos to recipients and identification of transgenic animals
among newborns. Only 1.3% and 10% of the newborns were
transgenic in sheep and pigs, respectively (Hammer et al.,
5   Nature 315:680-683 (1985), incorporated herein by reference),
and 5% of bovine fetuses were transgenic (Biery et al.,
Theriogenology 29:224 (1988), incorporated herein by
reference).

     Since the frequency of transgene incorporation in
10 mammals is often low, and their gestation time long, the
detection of transgene integration, e.g.,in the preimplantation
embryo would be highly desirable. Methods for early detection
of transgene integration would significantly decrease the
number of pregnancies required to produce a transgenic animal
15 and substantially increase the likelihood that an implanted
embryo will produce a transgenic animal. These methods would
thus allow for far more efficient production of transgenic
offspring, providing significant savings of time and resources.
Such methods would be especially important for those animals
20 for which very low or non-existent frequencies of transgenesis
have been obtained, e.g. bovine species. The present invention
fulfills these and other needs.

SUMMARY OF THE INVENTION

25   The present invention provides methods for the early
detection of integrated transgenes in the nuclear genome of
animal embryos.

     In one embodiment of the present invention, such
integrated transgenes are detected by performing in situ
30 hybridization on at least one cell from an embryo with a
nucleic acid probe substantially complementary to the
transgene. Generally, the embryo cell(s) used for in situ
hybridization are in interphase (G_0 or G_1) or metaphase, but
preferably metaphase. The presence of such an integrated
35 transgene is detectable as a discrete hybridization signal on
both sister chromatids of a chromosome in a metaphase spread
and is detectable in the nucleus of interphase cells as
discrete intense hybridization signals against a diffuse

background of non-specific staining. Such a method is
preferably nondestructive: a biopsy of at least one cell is
removed from the embryo to be analyzed by _in situ_
hybridization, and the resulting biopsied cell is viable. The
5   probe is typically labeled by biotinylation, and is preferably
long, at least 2 kilobases in length to provide optimum
sensitivity.

    In another method of early detection of transgene
detection in animal embryos, one takes advantage of the
10   differences in DNA methylation patterns in prokaryotes and
eukaryotes.   Integration and replication of a transgene
methylated in a prokaryotic fashion causes it to become
methylated in a eukaryotic fashion. Such a change can be
tested for by use of methylation sensitive enzymes. The
15   restriction site of Dpn I, is methylated in Dam$^+$ strains of _E._
coli or may be methylated _in vitro_ by dam methylase at N6 of an
adenine of its restriction site. When integrated and
replicated as part of the nuclear genome of an animal embryo,
its restriction site loses its prokaryotic methylation pattern,
20   and can no longer be cleaved by Dpn I.

    In one preferred method of the present invention,
nuclear DNA from at least one cell from said embryo is treated
with a restriction enzyme, such as Dpn I, capable of cleaving a
methylated restriction site and incapable of cleaving said
25   unmethylated restriction site, thereby producing restriction
fragments of said nuclear DNA. These restriction fragments are
amplified by PCR with PCR primers substantially complementary
to sequences on different strands of the transgene and flanking
the unmethylated restriction site. The amplified transgene may
30   be detected only if the restriction enzyme has not cleaved its
restriction sequence.

    Alternatively, one may employ inverse PCR, cleaving
the nuclear DNA of the embryo with a restriction enzyme
incapable of cutting within the transgene, producing
35   restriction fragments of said DNA; adding ligase to the
restriction fragments under conditions which result in self
ligation of the restriction fragments, thereby producing
circularized restriction fragments; amplifying the circularized

restriction fragments by inverse PCR, thereby producing an
amplified transgene; and detecting the amplified transgene.

The embryos tested by these methods are typically
bovine morulas at the 8 to 16 cell stage or are compacted

5   embryos that have developed past the 16 cell stage. It is
preferable to clone the animal embryo prior to testing for an
integrated transgene, such as by nuclear transfer or
propagation of cloned cleaved embryos (e.g., hemi-embryos,
infra).

10   Methods are also provided which additionally
determine the sex of the embryo tested by the methods above
described. Also embraced are embryos which have been produced
by any of the methods of the present invention.

15   DETAILED DESCRIPTION OF THE INVENTION
The present invention provides methods for early
detection of transgene integration in embryos which permit
implantation and development to maturity of transgenic embryos
so analyzed. Two basic approaches are provided: the first is

20   based on the polymerase chain reaction (PCR), the second on the
in situ hybridization of nuclear DNA from embryonic cells with
labeled probes. Each is described below in detail. These not
only allow the early and rapid detection of transgene sequences
in the cells of embryos into which transgene DNA has been

25   introduced, but allow one to determine whether such DNA is
truly integrated into the nuclear genome, giving rise to a
transgenic animal, or is nonintegrated DNA which will
eventually be lost. False positives, arising when
nonintegrated DNA is mistakenly identified as integrated

30   transgenes, would otherwise demand a significant expenditure of
time and resources. The power of these techniques is enhanced
when they are used in concert, preferably using PCR as an
initial detection method, followed by in situ hybridization as
a confirmatory method.

35

Cell and embryo culture; biopsies
Transgene sequences may be introduced into host cells
in a variety of ways well known in the art, including:

electroporation (Thomas and Capecchi, Cell 51:503-512 (1987) or
microinjection of the transgene into the pronuclei of
fertilized oocytes or nuclei of ES cells of the animal (Zimmer
and Gruss, Nature 338:150-153 (1989); transfection of ES cells
5   in culture by calcium phosphate precipitation (Gossler et al.,
Proc. Natl. Acad. Sci. USA 83:9065-9069 (1986); and infection
of zygotes with a retrovirus containing the transgene
(Jaenisch, <u>Proc. Natl. Acad. Sci. USA</u>, <u>73</u>, 1260-1264 (1976);
Robertson et al., Nature 323:445-448 (1986); all incorporated
10   herein by reference).

      Cells into which have been introduced transgene
sequences are thereafter cultured <u>in vitro</u> until a "pre-
implantation embryo" is obtained. Such pre-implantation
embryos preferably contain approximately 16 to 150 cells. The
15   8 to 32 cell stage of an embryo is commonly referred to as a
morula. Those pre-implantation embryos containing more than 32
cells are commonly referred to as blastocysts. Blastocysts are
generally characterized as demonstrating the development of a
blastocoel cavity, typically at the 64 cell stage. Methods for
20   culturing fertilized oocytes to the pre-implantation stage
include, for example, those described by Gordon et al., Meth.
Enzymol. 101:414 (1984); Hogan et al., in <u>Manipulating the
Mouse Embryo</u>, Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, N.Y. (1986) (for the mouse embryo); and Hammer et al.,
25   Nature, 315:680 (1985) (for rabbit and porcine embryos);
Gandolfi et al. J. Reprod. Fert. 81:23-28 (1987); Rexroad et
al., J. Anim. Sci. 66:947-953 (1988) (for ovine embryos); and
Eyestone et al., J. Reprod. Fert. 85:715-720 (1989); Camous et
al., J. Reprod. Fert. 72:779-785 (1984); and Heyman et al,
30   Theriogenology 27:5968 (1987) (for bovine embryos); all
incorporated herein by reference.

      Once the pre-implantation embryo stage is obtained, a
biopsy is obtained, i.e., at least one cell is removed from the
embryo. When equal division is used, the embryo is preferably
35   not cultivated past the morula stage (32 cells). Division of
the pre-implantation embryo (reviewed by Williams et al.,
Theriogenology 22:521-531(1986), incorporated herein by
reference) may result in two "hemi-embryos" (hemi-morula or

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hemi-blastocyst), at least one of which is viable, i.e.,
capable of subsequent development after implantation into the
appropriate female to develop in utero to term. It is to be
understood that such an embryo may be either equally or
5   unequally divided into two hemi-embryos which are not
necessarily of equal cell number. Indeed, only one or two
cells may constitute a biopsy from a single embryo; for that
reason, one may more accurately speak of a "biopsied embryo"
resulting from the removal of a biopsy of one or more cells
10   from an embryo for analysis by the early detection methods
provided herein. Essentially, all that is required is that one
of the embryos which is not analyzed as hereinafter described
be of sufficient cell number to develop to full term in utero.
        The biopsied embryo, if shown to be transgenic, may
15   be implanted in a female recipient animal to develop to term.In
a preferred embodiment of the present invention, the methods
for detecting transgenesis in pre-implantation embryos provided
herein are combined with embryonic cloning steps to generate a
population of transgenic embryos having the same genotype,
20   several of which may provide cell biopsies for transgenesis
detection. After transgenesis detection by the methods of the
present invention, such cloned embryos may thereafter be
implanted into recipient females to produce a population of
transgenic animals also having the same genotype. Since only
25   about 50% of biopsied embryos develop to birth upon transfer to
a recipient, embryonic cloning ensures not only that enough
biopsied cells are available to provide a conclusive test for
transgenesis, but also that at least one of the cloned embryos
shown to be transgenic will produce a transgenic animal.
30   Such embryo cloning may be performed by several
different approaches. In one cloning method, the transgenic
hemi-embryo is cultured in the same or in a similar media as
used to culture individual oocytes to the pre-implantation
stage. The "transgenic embryo" so formed (preferably a
35   transgenic morula) is then divided into "transgenic hemi-
embryos" which can then be implanted into a recipient female to
form a clonal population of two transgenic animals.
Alternatively, the two transgenic hemi-embryos obtained may be

again cultivated to the pre-implantation stage, divided, and
recultivated to the transgenic embryo stage. This procedure is
repeated until the desired number of clonal transgenic embryos
having the same genotype are obtained. Such transgenic embryos
5   may then be implanted into recipient females to produce a
clonal population of transgenic animals.

     In a preferred cloning method, the transgenic embryo
is cloned by nuclear transfer according to the techniques of
Prather et al. (Biol. Reprod. 37:59-86 (1988), incorporated
10 herein by reference) and Roble et al. (J. Anim. Sci. 64:642-664
(1987); incorporated herein by reference). According to this
method, nuclei from individual cells of the transgenic embryo
are transplanted into enucleated oocytes, each of which is
thereafter cultured to the blastocyst stage. At this point,
15 the transgenic embryos may be subjected to another round of
cloning or may be transferred to a recipient parent for
production of transgenic offspring having the same genotype.
By these means one may produce multiple, genetically identical
embryos. One may obtain one or a small number of cells from
20 each of these identical embryos to test for transgenesis by the
methods of the present invention.

     The term "transgenesis" is understood herein as
meaning the integration of a transgene into the nuclear genome
of a host organism, and its subsequent replication and stable
25 maintenance as part of the nuclear genome. By "integration" is
meant that the transgene is located on a chromosome, continuous
with and linked by covalent bonds to the double stranded DNA
which constitutes the chromosome. This situation may be
contrasted with that of non-integrated extra-chromosomal DNA
30 elements such as plasmids, episomes, and double minutes.

     It is to be understood that transgenic embryos and/or
transgenic animals having the same "genotype" means that the
genomic DNA is substantially identical among the individuals of
the embryo and/or transgenic animal population, having derived
35 from the same genetic source. During mitosis, however, various
somatic mutations may occur which may produce some variations
in the genotype of one or more cells and/or animals. Thus, a

population considered to have the same genotype may demonstrate
individual or subpopulation variation.

<u>Transgene detection using PCR</u>

A variety of PCR methods are well known and widely
practiced in the art. See, e.g., U.S. Patent Nos. 4,683,195
and 4,683,202, and <u>PCR Protocols: A Guide to Methods and
Applications</u>, Innis et al. eds., Academic Press, San Diego
(1990), all incorporated herein by reference. Reagents and
hardware for conducting PCR are commercially available through
Perkin-Elmer/Cetus Instruments (PECI) of Norwalk, Connecticut.

Briefly stated, PCR is a cyclical process for the
amplification of a nucleic acid template comprising the steps
of: (a) denaturation of the template (generally by heating to
95°C to 100°C), (b) hybridization of dual oligodeoxynucleotide
primers to the denatured template, and (c) template
replication, consisting of an extension of these primers by a
DNA polymerase (generally a thermostable polymerase such as Taq
polymerase). Among the other reagents necessary for PCR are
deoxynucleotide triphosphates (dNTPs, i.e., A, C, G, and T and
their analogues) and a buffer to provide, for example, the salt
and pH conditions necessary for optimal polymerase activity.
The primers are so designed that they hybridize to sequences
flanking the region to be amplified, one on each strand, and
that the single strand generated by extension of each primer
can act as a template for the extension of the other primer.
Thus, each cycle of replication provides a two-fold
amplification of the template. PCR can be thus be used to
amplify target DNA sequences several million-fold. The
amplified DNA can then be analyzed by restriction enzymes and
electrophoresis.

In the methods of the present invention,
oligonucleotides primers are synthesized that preferably
hybridize specifically to sequences in the transgene, or, if
present, to sequences in accompanying vector sequences. Upon
amplification of the target sequence a DNA fragment of specific
size will be generated.

A typical PCR buffer contains 10 to 50 mM Tris-HCl
(between pH 8.3 and 8.8) when measured at 20°C, 50 mM KCl and
1.5 mM $MgCl_2$. Modifications of this buffer may be necessary
for certain combinations of target nucleic acids and primers,

5   as will be readily appreciated by those skilled in the art.

A number of suitable commercially available DNA
polymerases are appropriate for performing PCR. Thermostable
polymerases are preferred, such as those obtained from Thermus
aquaticus (Taq polymerase) or T. flavis. For PCR or other

10   amplification procedures, or for DNA sequencing reactions,
other polymerases may be appropriate, including E. coli DNA
polymerase I or its Klenow fragment, reverse transcriptase,
phage T4 or T7 polymerases, or structural variants and modified
forms of these and other polymerases.

15   The sensitivity of PCR is particularly useful for
analyzing small DNA samples (e.g. from a small number of
cells). This sensitivity can, however, be problematic unless
stringent precautions are taken to avoid specific
contamination. DNA contamination from aerosol particles,

20   pipettes, primers, etc. can be a big problem when the DNA used
for injection of the embryos is prepared in the same lab where
PCR is performed. Furthermore, PCR cannot discriminate between
integrated DNA and non-integrated DNA. The following stringent
measures can prevent contamination: PCR analyses in a

25   completely isolated lab equipped with a flow hood with filter;
special equipment (pipettes, microfuges, etc.) dedicated to
PCR; all reagents (primers, buffers, Taq polymerase) supplied
in kits that can be opened and used only once.

The problem of discriminating integrated from non-

30   integrated DNA constructs may be solved by taking advantage of
the difference in methylation patterns between DNA replicated
in prokaryotes and DNA replicated in eukaryotes. DNA
constructs which have been cloned in prokaryotes and/or
methylated in vitro will retain the prokaryotic methylation

35   profile, unless they integrate in and, consequently, are
replicated as part of the host genome. For example, the A of
the sequence GATC, the recognition site for the restriction
enzyme Dpn I, is methylated in Dam[+] strains of E. coli; it is

unmethylated in eukaryotic cells, which lack the dam methylase.
Eukaryotic DNA, on the other hand, is methylated at G in the
sequence GC. After several cleavages, the integrated
transgene, having been replicated along with the nuclear
5   genome, will be methylated in a eukaryotic fashion. By
digestion prior to PCR with restriction endonucleases capable
of cutting methylated restriction sites but not nonmethylated
restriction sites (e.g., Dpn I), it should be possible to
eliminate nonintegrated DNA while leaving the integrated, i.e.
10   restriction endonuclease resistant, DNA available for
amplification. Thus, only embryos having an integrated
transgene should give rise to an amplified band. It should
also be noted that the use of such a restriction enzyme also
reduces the problem of contamination by bacterial DNA (e.g.,
15   plasmid) contamination.

        It will be apparent to those skilled in the art that
one may likewise employ other methylation sensitive enzymes
which can cut sequences in the transgene which display the
eukaryotic methylation pattern but which fail to cut the same
20   sequences when they display a prokaryotic methylation pattern.
For example, one may first amplify the transgene by PCR, then
digest the amplified DNA with such a methylation sensitive
enzyme having one or more restriction sites within the
transgene (i.e., between the sites to which the two PCR primers
25   hybridize). One would then detect on a stained gel or Southern
blot of the PCR amplification products two (or more) DNA
fragments resulting from the digestion of the single amplified
DNA product.

        An alternative method for detecting integrated
30   transgenes, "inverse PCR", makes use of primers which have
opposite (reverse) orientation (Triglia et al., Nucl. Acids
Res. 16:8186 (1988); Ochman et al., Genetics 120:621-623
(1988), both incorporated herein by reference). The DNA
isolated from biopsies is cut with restriction enzymes and the
35   fragments are circularized by ligation. In biopsies from non-
transgenic embryos, DNA fragments of expected length are
amplified (derived from concatamers), while DNA from transgenic
embryos will also give rise to fragments of varying size.

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These fragments contain host sequences which are present
between the reverse primers in the religated circles.

Since the DNA introduced into mammals ("transgene")
may be quite long (up to 50 kb or more, and typically 20 kb or
5   more) and often lack vector sequences, PCR primers are
preferably designed to be complementary to sequences within the
transgene itself.

A preferred method for detecting transgenesis by PCR
at an early stage in the embryo's development employs a
10 methylation sensitive restriction endonuclease such as Dpn I.
Dpn I recognizes and cleaves the sequence GATC in double
stranded DNA only when the adenine in each strand within this
sequence is methylated at N-6. In this preferred method, the
transgene containing the sequence GATC is methylated prior to
15 microinjection either by transferring the transgene on an
appropriate plasmid to a Dam$^+$ strain of a microorganism such as
E. coli MM294, or by directly methylating the transgene in
vitro with dam methylase (commercially available from a number
of vendors, including New England Biolabs). The methylated
20 transgene (preferably without any exogenous sequences such as
plasmid vector) is then microinjected into fertilized oocytes
(approximately 10 to 500 copies per pronucleus, more preferably
50 to 100 copies per pronucleus). The fertilized oocytes so
obtained are cultured in vitro to the pre-implantation stage.
25 During this early growth and cell division phase, the genomic
DNA is replicated. Accordingly, those copies of the methylated
transgene integrated into the genome of the fertilized oocyte
are unmethylated after replication, whereas any non-integrated
transgenes which may still exist after replication will remain
30 methylated (Lacks et al., J. Mol. Biol. 114:153 (1977),
incorporated herein by reference). This differential
methylation pattern for an integrated versus a non-integrated
transgene permits the identification of which fertilized
oocytes have integrated the transgene into the genome.
35 The identification of the pre-implantation embryos
containing the integrated transgene is achieved by analyzing
the DNA from each of the embryo biopsies (or the pooled
biopsies from several genetically identical embryos arising

from cloning a single embryo). Such DNA is typically obtained
by lysing the biopsied cells and analyzing the released DNA
after treatment as described by Ninomiya et al., Molecular
Reproduction and Development 1:242-248 (1989), incorporated

5   herein by reference. Each of the DNA samples is treated with
Dpn I. Thereafter, PCR is used to amplify all or part of the
transgene. When the entire transgene is amplified, two
extension primers each complementary to opposite strands at
opposing ends of the transgene are used. When, however, less

10   than the entire transgene is amplified, such extension primers
are chosen such that the amplified gene product spans the Dpn I
site in the transgene. That is, the primer binding sites for
the two PCR primers flank the Dpn I site. If Dpn I cleavage
has not occurred, PCR amplification will result in amplified

15   sequences having a predicted size, whereas no amplification
product will result for those transgenes which have been
cleaved. Generally, the Dpn I digested and PCR amplified DNA
from the biopsy is subjected to electrophoresis. Although it
is often possible to simply visualize the amplified DNA on an

20   ethidium bromide stained gel, electrophoresis is preferably
followed by hybridization with labeled probe complementary to
the region of the transgene between the two extension primers.
By these means the size of the amplified DNA sequences, if any,
is determined. The presence of an amplified sequence of the

25   appropriate size indicates whether the transgene has been
integrated into the pre-implantation embryo from which the
biopsy was obtained (now called a "transgenic biopsied
embryo").

       If integration is detected, the remaining untreated

30   transgenic biopsied embryo is transplanted into a recipient
parent. After in utero development the transgenic animal
having the desired phenotype conferred by the integrated
transgene is identified by an appropriate method in utero or
after birth.

35   The use of Dpn I, of course, requires that the
sequence GATC be present in the transgene of interest. In
those cases when such a sequence is not present, it may be
readily introduced into the transgene by site directed

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mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA 82:488 (1985),
incorporated herein by reference) or cassette mutagenesis
(Wells et al., Gene 34:315 (1985), incorporated herein by
reference), provided such mutagenesis does not change the amino

5   acid sequence encoded by the transgene (or causes an
inconsequential change in amino acid sequence) and that any
codons so generated are functional in the transgenic animal of
interest.

          In an alternate embodiment, the above described

10   method for detecting transgenesis in pre-implantation embryos
is combined with embryonic cloning steps to generate a
population of transgenic embryos having the same genome type
which may thereafter be implanted into recipient females to
produce a population of transgenic animals also having the same

15   genotype. In this regard it is to be understood that
transgenic embryos and/or transgenic animals having the same
"genotype" means that the genomic DNA is substantially
identical between the individuals of the embryo and/or
transgenic animal population. It is to be understood, however,

20   that during mitosis various somatic mutations may occur which
may produce some variations in the genotype of one or more
cells and/or animals. Thus, a population having the same
genotype may demonstrate individual or subpopulation
variations.

25   To detect the PCR amplified products, e.g., a sample
is electrophoresed on an agarose or polyacrylamide gel.
Smaller DNA fragments resulting from amplification are
preferably. analyzed by polyacrylamide gel electrophoresis. DNA
bands are visualized by ethidium bromide staining, or, to

30   increase sensitivity, gel electrophoresis can be followed by
Southern blotting or dot blotting and hybridization with a
labeled probe by techniques well known in the art. Also,
labeled PCR primers, which are incorporated into the amplified
DNA product, may be employed by methods well known in the art

35   to assist in visualization of the amplification product and
thus increase sensitivity.

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Transgene detection by in situ hybridization

Techniques have been developed in which nucleic acid
probes are used to locate specific complementary nucleic acid
sequences, e.g., specific DNA sequences on intact chromosomes,
in situ, a procedure called "in situ hybridization". Labeled
5   nucleic acid probes of a predetermined nucleotide sequence are
hybridized to the chromosomes of sample cells (or tissues), the
DNA strands of which have been denatured to a substantially
single-stranded character by a brief exposure to a very high pH
condition. The chromosomal regions that bind the probe(s)
10   during the hybridization step are examined by microscopy to
determine whether they hybridize to the probe(s) and thus
contain the specific nucleic acids of interest.

Beside the use of radionuclide-labeled nucleic acid
15   probes, there are two distinct non-radioactive hybridization
methods: the direct and the indirect methods. In the direct
methods, the reporter molecule is bound to the nucleic acid
probe so that the molecular hybrids between probe and target
sequences can be visualized microscopically immediately after
20   the in situ hybridization procedure. Such methods include the
terminal fluorochrome labeling procedure of RNA probes (Baumann
et al., 1980, 1984, which are incorporated herein by reference)
and the direct enzyme labeling procedure of nucleic acids (Renz
and Kurz (1984, which is incorporated herein by reference).

25   For indirect procedures, the probe must contain an
element, introduced chemically or enzymatically, that renders
it detectable by affinity cytochemistry, hence the term
indirect. Again, the presence of such an element should not,
or only in a limited way, interfere with the actual
30   hybridization reaction and the stability of the resulting
hybrid. A number of such hapten modifications, including
acetylaminofluorene, mercury, biotin and transamination
procedures, have been described (Langer et al., Proc. Natl.
Acad. Sci. USA 78:6633-6637 (1981); Leary et al., Proc. Natl.
35   Acad. Sci. USA 80:4045-4049 (1983); Landegent et al., Exp. Cell
Res. 153:61-72 (1984); Tchen et al., Proc. Natl. Acad. Sci. USA
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et al., Nucl. Acids Res. 14:6471-6488 (1986); Shroyer and
Nakane, J. Cell Biol. 97:377a (1983); Van Prooijen-Knegt et
al., Exp. Cell Res.141:397-407 (1982); Raap et al.,
Histochemistry 81:517-520 (1984); Viscidi et al., J. Clin.
5   Microbiol. 23:311-317 (1986); Rudkin and Stollar, Nature
265:472-473 (1977); see for review Raap et al., In *Techniques
in Immunochemistry*, vol. IV, Bullock and Petrusz, eds., New
York, Academic Press, pp. 167-197 (1989); all incorporated
herein by reference). When antibodies against the reporter
10   molecules are available, then direct methods are also amenable
to immunocytochemical amplification (Bauman et al.,
Histochemistry 73:181-193 (1981); Lansdorp et al., J.
Histochem. Cytochem. 32:172-178 (1984); Pinkel et al., Proc.
Natl. Acad. Sci. USA 85:2934-2938 (1986); all incorporated
15   herein by reference).
            Recently, the chemical synthesis of oligonucleotides
containing functional groups (e.g. primary aliphatic amines or
sulfhydryl groups) has been described, thus allowing in
principle the applicaction of nucleotide oligomers in non-
20   radioactive *in situ* hybridization by coupling haptens (e.g.,
biotin or digoxygenin) or reporters like fluorochromes or
enzymes (Agrawal et al., Nucl. Acids Res. 14:6227-6245 (1986);
Chollet and Kawashima, Nucl. Acids Res. 13:1529-1541 (1985);
Haralambidis et al., Nucl. Acids Res. 12:4857-4876 (1987);
25   Jablonski et al., Nucl. Acids Res. 14:6115-6129 (1986); Kempe
et al., Nucl. Acids Res. 13:45-57 (1985); Ruth, DNA 3:123-129
(1984); all incorporated herein by reference).
            Labeling DNA probes chemically instead of
radioactively improves the spatial resolution of this
30   technique. Preferably, the probes are synthesized with
nucleotides that contain a biotin side chain, and the
hybridized probes are detected by staining with a network of
streptavidin and some type of marker molecule. With this
technique it is possible to detect specific, single copy
35   nucleic acid sequences in individual cells and on individual
chromosomes (See for review: Raap et al., In *Techniques in
Immunocytochemistry*, vol. IV, ed. G. Bullock and P. Petrusz:

New York, Academic Press (1990), incorporated herein by
reference).

    The advantage of the use of metaphase chromosomes for
in situ hybridization is that it facilitates the discrimination
5   between integrated and non-integrated DNA by localizing
specific hybridization signal(s) indicative of integrated
transgene DNA on adjacent chromatids. Alternatively, in situ
hybridization can be performed on cells which are not in
metaphase (e.g., interphase cells from an embryo biopsy, which
10 are typically not synchronized) and integrated transgene DNA
can be identified by the presence of discrete hybridization
signal(s) which can be differentiated from the non-specific
hybridization background. A discrete hybridization signal is
identified as one or more localized intense staining positions
15 which are differentiated from non-specific hybridization by
visual inspection, autoradiographic analysis, or computerized
imaging methods wherein background noise is subtracted by one
or more imagen analysis algorithms known in the art.
Nonintegrated DNA molecules will probably be present in high
20 numbers, fused together in one or a few large concatamers, yet
diffused randomly throughout the nucleus and not associated
directly with a chromosome. Integrated DNA will be visualized
as discrete spots in the nucleus of cells (generally fixed
cells), and in metaphase cells can be visualized as discrete
25 spots on one or both sister chromatids at the same location for
each integration site.

    Initially, in situ hybridization methods used DNA or
RNA probes labeled directly with a terminal fluorochrome. The
sensitivity of these direct methods was low. Therefore,
30 immunocytochemical amplification procedures have been developed
to increase detection sensitivity. Several chemical and
enzymatic nucleic acid hapten modifications are now available
for the amplification on DNA or RNA probes for in situ
hybridization (Raap et al., 1989). All modifications have in
35 common that the hapten does not affect the hybridization
properties of the probes. Most haptenized probes are detected,
after hybridization, with antibodies specific for the hapten.

Although _in situ_ hybridization is a fairly complex
multistep procedure in which each step must be optimized, the
technique has been successfully employed, for example, in the
diagnosis of viral infection (Brigati et al., Virology 126:32-
5   50 (1982); Burns et al., J. Clin. Pathol. 39:1066-1073 (1986);
Raap et al., Histochemistry 88:367-373 (1988); all incorporated
herein by reference) and the analysis of gene expression at the
mRNA level (Rudkin and Stollar, Nature 265:472-473 (1977),
incorporated herein by reference); for the assessment of
10   chromosome copy number in early prenatal diagnosis (Julien et
al., Lancet ii:863-864 (1986), incorporated herein by
reference) and karyotyping of tumor cells (Devilee et al.,
Cancer Res. 48:5825-5830 (1988); Hopman et al., Histochemistry
89:307-316 (1988); all incorporated herein by reference).
15   Heretofore, however, it has not been employed for the detection
of incorporated transgenes in early embryos.

Currently, the sensitivity of the haptenized probes
is sufficient for single copy gene localization on metaphase
nuclei (Albertsen, EMBO J. 4:2493-2498 (1985); Bhatt et al.,
20   Nucleic Acids Res. 42:42:153-161 (1988); Landegent et al.,
Nature 317:175-177 (1985); Lawrence et al., Cell 52:51-61
(1988); all incorporated herein by reference).

Further testing to confirm transgenesis and sex determination

25   In a preferred embodiment of the present invention,
PCR is employed to provide an initial determination whether the
transgene is incorporated into the genome of an embryo, while
in situ hybridization is used to confirm the results of the
initial testing. Alternatively, PCR is used to confirm the
30   results obtained by an initial test using _in situ_
hybridization. The sex of the embryos so tested can further be
sexed by PCR or _in situ_ hybridization using probes specific for
sex-determining sequences, such as sequences characteristic of
the Y chromosome or other sequences involved in sex
35   determination (e.g., sex steroid receptor genes, etc.) which
are not linked to the Y chromosome and which are known in the
art.

To follow up and provide further confirmation for the
methods of the present invention for detecting early
transgenesis, other methods may be used, including _in utero_ and
post partum analysis of tissue. _In utero_ analysis is performed

5   by several techniques. In one such technique, transvaginal
puncture of the amniotic cavity is performed under echoscopic
guidance (Bowgso et al. (1975) Bet. Res. 96:124-127; Rumsey et
al. (1974) J. Anim. Sci. 39:386-391; both incorporated herein
by reference). This involves recovering about 15 to 20

10 milliliters of amniotic fluid between about day 35 and day 100
of gestation. This volume of amniotic fluid contains about
1000 to 12,000 cells per ml originating from the urogenital
tract, the skin, and possibly the lungs of the developing
embryo. Most of these cells are dead. Such cells, however,

15 contain genomic DNA which is subjected to PCR analysis for the
transgene as an indication of a successful transgenesis.
Alternatively, fetal cells may be recovered by chorion
puncture. This method also may be performed transvaginally and
under echoscopic guidance. In this method, a needle is used to

20 puncture the recipient animal's placenta, particularly the
placentonal structures, which are fixed against the vaginal
wall. Such sampling may be performed around day 60 of
gestation. Chorion cells, if necessary, are separated from
maternal tissue and subjected to PCR analysis for the transgene

25 as an indication of successful transgenesis.

Transgenesis may also be detected after birth. In
such cases, transgene integration can be detected by taking an
appropriate tissue biopsy such as from the ear or tail of the
putative transgenic animal. About one to two centimeters of

30 tail or about five to ten square millimeters of ear are
obtained, followed by Southern blotting with a probe for the
transgene according to the method of Hogan et al. (1986)
Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory,
incorporated herein by reference.

35

Recombinant DNA Techniques

It will be readily apparent that the present
invention relies heavily upon a thorough knowledge and

understanding of recombinant DNA technology. The recombinant
DNA techniques employed when using the present invention are
well established and constitute recognized methods, e.g., for
the use of restriction endonucleases; for the preparation of

5 predetermined nucleotides in sequence as hybridization probes;
and for the various methods of labelling such DNA or RNA probes
using a variety of labels, such as radionuclides. Accordingly,
it is presumed that one practicing the present invention is
familiar with the applications and limits of the various

10 techniques and will recognize that minor changes in reagents,
concentrations, temperature, reaction times and similar
alterations of known methods are merely obvious variations of
choice. Any major differences from the published and well
accepted techniques will be identified and described in detail

15 as necessary. However, no description or repetition of the
many compositions, protocols and manipulative techniques will
be given here for these well known procedures. A complete
description and recitation of the theory and practice of
various recombinant DNA techniques, as well as various

20 reagents, experimental parameters and other practical
considerations, one may consult, <u>inter alia</u>, Sambrook et al.,
<u>Molecular Cloning: A Laboratory Manual</u> (2nd ed.), Vols. 1-3,
Cold Spring Harbor Laboratory, (1989) or <u>Current Protocols in
Molecular Biology</u>, F. Ausubel et al., ed. Greene Publishing and

25 Wiley-Interscience, New York (1987), which are incorporated
herein by reference.

       In addition it is presumed and understood that the
terminology, technical and otherwise, used herein follows their
usual, well understood meanings as they are used and applied in

30 common parlance or the technical literature. For these
reasons, only if a term has not been used previously within the
field generally or if an unusual definition is employed, then
and then only will a formal definition be provided as part of
the text. For this reason, such technical terms as nucleotide,

35 nucleic acid, genome, vector, and similar technical terms are
presumed to be commonly understood by the practitioner
ordinarily skilled in this art.

As used herein, the term "transgene" refers to a DNA
sequence which is capable of producing a desirable phenotype
when contained in the genome of cells of a transgenic animal.
Such a transgene often comprises a recombinant DNA sequence

5   encoding a "recombinant polypeptide". In such cases, the
transgene is capable of being expressed to produce the
recombinant polypeptide. Unless otherwise noted, the term also
is used to embrace vector sequences and non-coding flanking
sequences which may accompany such polypeptide-encoding

10 sequences.
          Specific hybridization is defined herein as the
formation of hybrids between a probe polynucleotide (e.g., a
polynucleotide of the invention which may include
substitutions, deletion, and/or additions as compared to a

15 target sequence) and a specific target polynucleotide (e.g., a
polynucleotide having a predetermined sequence, such as a
transgene sequence), wherein the probe preferentially
hybridizes to the specific target such that, for example, a
single band corresponding to a transgene restriction fragment

20 can be identified on a Southern blot of DNA prepared from a
cell having an integrated copy of the transgene DNA.

PCR Primers and Nucleic Acid Probes
          The PCR primers used in the methods of the present

25 invention are oligonucleotides, whether occurring naturally as
in a purified restriction digest, or produced synthetically,
which are capable of hybridizing specifically to a known
sequence in a target gene. When a PCR primer, especially the
terminal 3' nucleotide of the primer, has hybridized, it acts

30 as a point of initiation of synthesis under conditions in which
synthesis of a primer extension product is favored. Such
conditions typically include the presence of four different
nucleotide triphosphates and a thermostable polymerase in an
appropriate buffer and at a suitable temperature.

35   Such primers are preferably single stranded for
maximum efficiency and amplification, although double stranded
primers may be employed if denatured to separate the
complementary strands before use. The oligonucleotides

employed as primers may contain naturally-occurring nucleotides
or their analogs, such as 7-deazaguanosine or inosine, and may
be either DNA or RNA.

     Such an oligonucleotide will preferably contain at
5   least one or preferably more than one region of eight or more
consecutive nucleotides having perfect complementarity with the
target sequence. They will also usually have one or more
nucleotides at the 3' end displaying perfect complementarity
(i.e., base pairing) with a single-stranded region of a target
10 nucleic acid. However, longer oligonucleotides (e.g., more
than 20 nucleotides) can anneal with the desired specificity,
even though no such region of consecutive nucleotides with
perfect complementarity may be present. In general,
oligonucleotides that specifically hybridize to a predetermined
15 sequence (unique or repetitive) or sequence motif are suitable
for use as primers.

     The primer sequence need not, however, reflect the
exact sequence of the template, and may include sequences in
addition to those allowing the primer to hybridize with
20 specificity to the template. Alternatively, noncomplementary
bases or longer sequences can be interspersed into the primer
provided that the primer sequence has sufficient
complementarity with the sequence of the target sequence to
hybridize with it and thereby allow synthesis of the extension
25 product. In addition, the primer may include nucleotides which
have been substituted, e.g., with biotin. As a consequence of
amplification by PCR, the sequence and substituents of the
primer are introduced into the amplified product.

     Once the appropriate primer sequences are determined,
30 they are preferably synthesized using commercially available
methods and equipment. Synthetic oligonucleotides can be
produced by the solid phase phosphoramidite method according to
Caruthers et al., Cold Spring Harbor Symp. Quant. Biol.,
47:411-418 (1982), and Adams et al., J. Am. Chem. Soc., 105:661
35 (1983), or the triester method according to Matteucci, et al.,
J. Am. Chem. Soc., 103:3185 (1981), all incorporated herein by
reference.

Preferably the oligonucleotide is an
oligodeoxyribonucleotide. Its exact length will depend on such
factors as number of mismatches, if any, temperature, salt
conditions, and other parameters discussed above.

5   Oligonucleotide primers typically are from about 8 to 50,
usually about 12 to 50, and preferably 16 to 30 nucleotides in
length, although longer or shorter primers may be appropriate.

Suitable probes, e.g., for in situ analysis or for
analyzing Southern blots of the products of PCR amplification,

10 may be RNA, DNA, or an analogous polymer (e.g., a polyamide
nucleis acid or a methylphosphonate oligonucleotide) and may be
either double-stranded or single-stranded. In situ
hybridization probes will typically be at least about 100 to
1000 bases long, preferably greater than 2 kb in overall length

15 to increase hybridization signal as long as possible (greater
than 2 kb is preferable) to increase sensitivity of detection
(e.g., signal-to-noise ratio), although shorter probes may be
used, and will preferably be complementary to unique sequences
in a transgene (except where the transgene comprises repetitive

20 sequences that may be distinguished from sequences in the host
genome by specific hybridization) to reduce the chance for
nonspecific hybridization. Probes for Southern blots may be
shorter probes substantially complementary to sequences in a
transgene. The nucleic acid probes may be derived from genomic

25 DNA or cDNA, prepared by chemical or enzymatic synthesis (e.g.,
as an RNA transcribed from a template sequence comprising a
sequence substantially complementary to a transgene), or may be
a hybrid of the various combinations. Recombinant nucleic
acids comprising sequences otherwise not naturally occurring

30 may also be employed. They may contain naturally occurring
nucleotides or their analogs, such as 7-deazaguanosine or
inosine.

Such probes may be labeled by any of the methods
commonly used in the art, such as nick translation or random

35 hexamer labeling. Such nucleic acid probes will include an
isolated nucleic acid attached to a label or reporter molecule.
Probes may be prepared by nick translation, Klenow fill-in
reaction, random hexamer priming, or other methods known in the

23

art. For isolating nucleic acids, choosing label or reporter
molecules, labeling probes, and other aspects of probe
preparation see, <u>inter alia</u>, Sambrook et al., <u>Molecular</u>
<u>Cloning: A Laboratory Manual</u> (2nd ed.), Vols. 1-3, Cold Spring

5   Harbor Laboratory, (1989), <u>Current Protocols in Molecular</u>
<u>Biology</u>, F. Ausubel et al., ed. Greene publishing and Wiley-
Interscience, New York (1987), incorporated herein by
reference.

10 <u>Substantial homology or complementarity; Selective and</u>
<u>stringent hybridization</u>
          An oligonucleotide primer or a probe is
"substantially homologous" or "substantially complementary" to
a target nucleic acid when it preferentially anneals or

15 hybridizes to a desired sequence position on a strand of the
nucleic acid being targeted or its complementary strand such
that stable and specific binding occurs between the primer and
the target nucleic acid under selective conditions (<u>See</u>, M.
Kanehisa, <u>Nucleic Acids Res.</u> 12:203 (1984), incorporated herein

20 by reference). Selectivity of hybridization exists when
hybridization occurs which is more selective than total lack of
specificity, and is generally marked, in the methods of the
present invention, by the formation of a desired extension
product.

25   Proper annealing conditions depend, for example, upon
an oligonucleotide's length, base composition, and the number
of mismatches and their position on an oligonucleotide, or on
the melting temperature (T_m of the oligonucleotide), salt
concentration of the medium, and other conditions, and

30 generally are determined according to formulae and methods
known in the art, which can include generation of a
standardization of calibration hybridization temperature curve.
For discussions of oligonucleotide design and annealing
conditions, see, for example in Sambrook et al. (1989); Berger

35 and Kimmel, <u>Methods in Enzymology, Volume 152, Guide to</u>
<u>Molecular Cloning Techniques</u> (1987), Academic Press, Inc., San
Diego, CA; and F. Ausubel et al., ed. (1987), which are
incorporated herein by reference.

Stringent primer annealing conditions will vary with the specific application, but typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and preferably less than about 200 mM.

5 Temperature conditions will typically be greater than 22°C, more typically greater than about 30°C and preferably in excess of about 37°C. As other factors may dramatically affect the stringency of hybridization, including base composition and size of the complementary strands, the presence of such salts

10 as MgCl$_2$, the presence and concentration of formamide, the presence and concentration of dextran sulfate, and the extent of base mismatching between probe and target, the combination of parameters is more important than the absolute measure of any one alone.

15 Alternatively, on the basis of sequence comparison, an oligonucleotide primer or a probe is considered "substantially homologous" or substantially complementary" to a target sequence whenever the primer and target sequence, or their complementary strands, when optimally aligned and

20 compared, are identical with appropriate nucleotide insertions or deletions, generally in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98 to 99.5% of the nucleotides. The terms "substantial similarity" or "substantial complementarity"

25 as used herein denotes a characteristic of a polypeptide sequence or nucleic acid sequence, wherein the polypeptide sequence has at least 50 percent sequence identity compared to a reference sequence, and the nucleic acid sequence has at least 70 percent sequence identity compared to a reference

30 sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 25 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a transgene construct (e.g., selectable marker sequence,

35 homologous recombination sequence, structural gene sequence); however, the reference sequence is at least 18 nucleotides long in the case of polynucleotides, and typically is longer than about 30 nucleotides or more.

25

The invention will better be understood by reference
to the following examples, which are intended to merely
illustrate the best mode now known for practicing the
invention, but the invention is not to be considered limited
5   thereto.

                        EXPERIMENTAL EXAMPLES

        Although the following examples are described in
detail for embryonic cells, it is believed that the method(s)
10   are generally applicable to detection of transgenesis in other
eukarotic cell types, particularly various types of somatic
mammalian cells such as nucleated cells of the
lymphohematopoietic lineage, fibroblasts, basal epithelial
cells, and others. The method(s) may also be used for sex
15   determination of embryos by using probes that detect the
presence of the Y chromosome and/or other non-Y-linked
sequences that are responsible for or correlate with sex
determination.

20   1.   In vitro Maturation, Fertilization and Culture of Bovine
Oocytes

        Immature oocytes are obtained in large quantity (400-
600/day) by aspirating follicles of ovaries obtained at
abbatoirs. Immature oocytes are cultured for a period in vitro
25   before they are competent to be fertilized. Once "matured",
oocytes are fertilized with sperm which has also been matured,
or "capacitated" in vitro. The pronuclei of the fertilized
oocyte is then injected with the transgene encoding for the
expression and secretion of human lactoferrin. Zygotes
30   resulting from this in vitro fertilization and microinjection
are then cultured to the late morula or blastocyst stage in
media with somatic tisssue, or in medium "conditioned" by
somatic tissue. Blastocysts are then transferred non-
surgically to recipient cattle for the balance of gestation or
35   analyzed for integration of the transgene as described herein.
        In vitro maturation (IVM). Ovaries are obtained
immediately after slaughter at local abbatoirs and oocytes are
recovered. Alternatively, oocytes are obtained from living

cattle by surgical, endoscopic, or transvaginal ultrasonic
approaches. In all cases, oocytes are aspirated from ovarian
follicles (2-10 mm diameter). After washing, oocytes are
placed in a maturation medium capable of supporting nuclear and
5   cytoplasmic maturation of bovine oocytes. Examples of such
media are given by Sirard et al. (Biol. Reprod. 39:546-552
(1988), incorporated herein by reference).

            In vitro fertilization (IVF). Matured oocytes are
fertilized with either fresh or frozen thawed sperm. Sperm are
10   then added to a fertilization media consisting of a modified
Tyrode's solution (Parrish et al. (1986) supra., incorporated
herein by reference) supplemented with heparin to induce sperm
capacitation (Parrish et al., Biol. Reprod. 38:1171-1180
(1988), incorporated herein by reference). Capacitation
15   constitutes the final sperm maturation process which is
essential for fertilization. Sperm and oocytes are co-cultured
for 18 hours. A useful feature of this IVF method is that (in
the case of frozen sperm) consistent, repeatable results are
obtained once optimal fertilization conditions for a particular
20   ejaculate have been defined (Parrish et al. (1986) supra.,
incorporated herein by reference).

            In vitro culture (IVC). Conventional culture
systems, which support development of mouse, rabbit, or human
ova, do not support development of bovine embryos past the 8-16
25   cell stage. This problem has been overcome by pre-conditioning
culture media with oviductal tissue. Oviduct-conditioned
medium will support bovine embryos past the 8-16 cell stage to
the blastocyst stage in vitro (Eyestone and First, J. Reprod.
Fert. 85:715-720 (1989), incorporated herein by reference).

30   Bovine embryos generally have proven refractory to in
vitro culture. This in part stems from the existence of a
"block" to cleavage in vitro at the 8-16 cell stage. This
block may be alleviated by culturing embryos in the oviducts of
rabbits (reviewed by Boland, Theriogenology 21:126-137 (1982),
35   incorporated herein by reference) or sheep (Willadeen in:
Mammalian Egg Transfer (E. Adams, ed.), pp. 185-210 (1982);
Eyestone et al., Theriogenology 28:1-7 (1987); both
incorporated herein by reference). However, these in vivo

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alternatives have been less than ideal, in that: (1) they
require the maintenance of large numbers of recipient animals,
(2) they require surgery to gain access to the oviducts for
transfer, and a second surgery (or sacrifice) to recover the
5   embryos, (3) all transferred embryos are seldom recovered, and
(4) access to embryos during culture for observation or
treatment is entirely precluded. The lack of in vitro culture
systems has hampered the development of various manipulation
techniques (such as gene transfer by pronuclear injection) by
10 preventing accumulation of basic information of the chronology
and ontogeny of bovine development, and by complicating the
process of culturing embryos to a stage compatible with non-
surgical embryo transfer and cryopreservation techniques (e.g.,
late blastocyst stages).

15   Bovine embryos did not yield to attempts to culture
them in vitro past the 8-16 cell "block" until Camous et al.,
J. Reprod. Fert. 72:479-485 (1984), incorporated herein by
reference) demonstrated cleavage to 216 cells when embryos were
co-cultured with trophoblastic tissue.

20   The co-culture procedure was extended to oviductal
tissue, based on the ability of homo- or hetero-oviducts to
support development from zygote to blastocyst. Thus, bovine
embryos co-cultured with oviductal tissue, or in medium
conditioned by oviductal tissue, developed from zygote to
25 blastocyst in vitro (Eyestone and First, J. Reprod. Fert.
85:715-720 (1989); Eyestone, "Factors affecting the development
of early bovine embryos in vivo and in vitro." Ph.D. Thesis,
University of Wisconsin (1989), both incorporated herein by
reference). Blastocysts have been produced in this system
30 after superovulation and artificial insemination, or by in
vitro maturation (IVM), and fertilization (IVF) of immature
oocytes. Blastocysts produced in this fashion resulted in
pregnancies and live calves after transfer to recipient
animals. The results obtained were as follows:

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40

28

Step	Efficiency (%)	Number (per 100)
IVM	90	90
IVF	80	72
IVC	30	22
Embryo transfer (% pregnant)	50	11

Therefore, from an initial daily harvest of 500 oocytes, it is expected that, on average, approximately 55 pregnancies will result.

Preparation of Oviduct Tissue and Use For Co-Culture and Conditioned Medium. Ovine oviducts are obtained after slaughter or by salpingectomy. The lumenal tissue is harvested by scraping an intact oviduct gently with a glass slide and washed five times in 10 ml modified Tyrode's-Hepes solution (Parrish et al., Biol. Reprod. 38:1171-1180 (1988), incorporated herein by reference). The final tissue pellet is suspended in M199 + 10% fetal calf serum at a ratio of 1 volume tissue to 50 volumes of media.

The tissue suspension can be used for embryo-co-culture. Alternatively, media may be conditioned for 48 h, and after centrifuging the suspension, the supernatant may be used as embryo culture medium. Conditioned medium may be stored at -70°C, if desired. Conditioned medium should be used at full strength for embryo culture (no dilution) (Eyestone (1989)).

2. Microinjection of hLF Transgene into Bovine Pronuclei

The DNA fragment containing the hLF expression unit is excised from the vector by digestion with the appropriate restriction enzyme(s) and separated by agarose gel electrophoresis. The fragment is purified by electroelution, phenol and chloroform extraction and ethanol precipitation. The DNA fragment is dissolved in and dialyzed in 10 mM Tris, 0.1 mM EDTA (pH 7.2) at a concentration of 1 to 2 μg/ml. Microinjection needles are filled with the dialyzed DNA solution.

29

Before _in vitro_ fertilization, cumulus cells are
removed from the egg by either vortexing at maximal speed for
two minutes or pipetting up and down several times. Bovine
pronuclei are injected in the same manner as murine pronuclei
5   (Hogan et al., in: _Manipulating the Mouse Embryo_, Cold Spring
Harbor Laboratory (1986), incorporated herein by reference)
with an additional centrifugation step in order to visualize
the pronuclei. The injection takes place 18-24 hours after
fertilization. The time varies depending on the bull used as a
10   source of semen. Nuclei become visible at different times in
different batches of semen.

Bovine oocytes, matured and fertilized _in vitro_, are
spun in a microfuge tube (Eppendorf) in 1 ml of Tyrode's-Hepes
solution (Parrish (1987), incorporated herein by reference) at
15   14500xg for eight minutes (Wall et al., Biol. Reprod. 32:645-
651 (1985), incorporated herein by reference). The embryos are
transferred to a drop of Tyrode's-Hepes solution on a
microscope slide covered with paraffin oil. Using a hydraulic
system the oocytes are fixed to the egg holder in such a way
20   that both the pronuclei are visible (using interference-
contrast or phase contrast optics). If necessary, the oocytes
are rolled to change their position on the egg holder to
visualize the pronuclei. The injection needle is brought into
the same sharp focus as one of the pronuclei. The needle is
25   then advanced through the zona pellucida and cytoplasm into the
pronucleus. A small volume, 1-3 pl, is injected (containing
20-100 DNA copies) into the pronucleus either by using a
constant flow or a pulse flow (using a switch) of DNA solution
out of the needle. Alternatively, two cell stage embryos are
30   spun as described and the nuclei of both blastomeres are
injected as described. The injected embryos are then
transferred to a drop of co-culture medium as described in
Example 1 in order to develop to the morula or blastocyst
stage.
35

3. Biopsies from developing embryos

In order to reproducibly and efficiently produce high
quality embryos, oviduct cell conditioned medium was compared

with oviduct epithelial cell coculture system as systems for
culturing in vitro matured and fertilized oocytes up to the
blastocyst stage. In the oviduct epithelial cell coculture
system, embryos are cultured on a monolayer of bovine oviduct
5   epithelial cells (BOEC). Using this system, 30% of the
fertilized oocytes develop to the blastocyst stage.

        The isolation of single or multiple blastomeres from
a preimplantation embryo (e.g., murine or bovine) requires
penetration of the zona pellucida (ZP). The murine ZP can
10   easily be dissolved at a precise, limited area using a constant
narrow flow of acidic Tyrode (pH 2.3) solution, although the ZP
of bovine embryos cannot be dissolved by acidic Tyrode, even
with a pH lower than 2.1.

        Three other mechanical methods for ZP penetration
15   have been tried to obtain biopsies from developing embryos:
(1) splitting the complete embryo with very sharp blades;
(2) making a hole in the ZP and pushing out single or multiple
blastomeres; and 3) making a slit in the ZP and aspirating
blastomeres with a beveled pipet.

20   (1) Splitting. This method is performed on later
stage pre-implantation embryos, e.g., morulae and blastocysts.
Cloned cleavage embryos have many applications, including the
use of a cloned cleavage embryo as a proxy for use in testing
procedures, such as many ISH protocols, which are not generally
25   compatible with preservation of embryo viability. The
advantages of this method are that it is very fast and simple
and gives a relatively large number of cells for analysis. The
disadvantages are that it is very crude method and a
significant number of embryos will not survive. Also, the
30   remaining part of the embryo has to be developed without ZP,
which could be troublesome for continued culture, as the embryo
tends to stick to the petri dish and/or BOEC monolayer), and in
the transfer procedure.

        The two other procedures are performed on 8 to 16
35   cell stage embryos. In these embryos the compaction process
has not taken place and individual blastomeres can still be
distinguished. For the isolation of blastomeres, the embryos
do not need to be incubated in cytochalesin in order to disrupt

cell-cell contracts. These procedures are performed as
follows:

        (2) Making a hole in the ZP and pushing out
blastomeres. With a sharp glass needle a hole is made in the
ZP and blastomere(s) can be pushed out using a blunt, polished
glass rod. This method is also very fast and has some elegance
in that the pushed out blastomere(s) will stick to the ZP on
the outside and can undergo several cleavages. This
facilitates the identification of blastomeres and subsequent
cleavage increases the number of cells that can be analyzed.
The disadvantage of this method is that the pushing out of
cells is less precisely controlled: multiple cells or only
parts of a single blastomere with or without a nucleus may be
pushed out.

        (3) Making a slit in the ZP and aspirating
blastomeres. In a preferred method, a slit is made in the ZP
which can be opened with a beveled glass pipet with an opening
large enough to subsequently aspirate blastomeres. After
withdrawing the aspiration pipet the ZP will reclose. This
method is more laborious but can be more precisely controlled
and causes the least disturbance of normal development. Little
damage is caused to the embryo and to the blastomeres that are
removed. Alternatively, an opening can be made in the ZP with
a sharp glass needle and one or more blastomeres can then be
aspirated out using a hollow micropipette which is inserted
into the opening made by the glass needle.

        These biopsy techniques are combined with the
transgene detection methods described below. Biopsies obtained
as described herein are also useful for multiplication of
embryos proven to be transgenic.

4.   Early Detection of Transgenesis with hLF Transgene
        Upon the microinjection of a construct, the oocyte is
cultured, preferably at least to the 8-cell stage, even though
analysis of embryos before that time is possible. Each embryo
is cleaved and subjected to lysis (King et al., Molecular
Reprod. and Devel. 1:57-62 (1988); proteolysis (Higuchi,

"Amplifications (A Forum for PCR Users" 2:1-3 (1989); both
incorporated herein by reference) and Dpn I digestion.
PCR is performed as described previously (Ninomiya et al.,
Molecular Reprod. and Devel. 1:242-248 (1989), incorporated
5   herein by reference) with sets of two primers which flank a
Dpn I site in the construct. One such set of primers are the
forward primer ATG AAA CTT ATC CTC ACC TGT CTT GTG (in the αS1
portion) and the reverse primer GGG TTT TCG AGG GTG CCC CCG AGG
ATG GAT (in the hLF portion) of an hLF transgene disclosed in
10   the commonly owned co-pending application U.S.S.N. 619,131,
incorporated herein by reference.

5.   Early Detection of Transgenesis in Murine Embryos by PCR
Analysis

15   72 hour-old murine embryos resulting from a mating
between a transgenic male and a nontransgenic female were
isolated. Two different males were used: one with a high
transgene copy number (40-60) and the other with a low copy
number (3). The number of cells in these embryos was around
20   50. About 50% of the embryos yielded a positive signal after
PCR amplification.
        To embyronic cells from blastocysts or complete
embryos in PBS (<5µl) are added 25µl H_2O, and the cells are
incubated 10 min at 95°C, then cooled slowly to room
25   temperature. Depending on the number of cells use all or part
of the sample is used in the following PCR reaction.
        The following buffer is added to a total volume of
50µl in a 500µl microfuge (Eppendorf) tube: 50 mM KCl, 10 mM
Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1%
30   Triton X-100, 50 µM each of dATP, dGTP, dCTP and dTTP, 50 pmol
forward primer, 50 pmol reverse primer, 0.8 U DpnI (Bethesda
Research Laboratories), and 2 U Taq polymerase (Promega). The
mixture is overlaid with a drop of light mineral oil to prevent
evaporation. The mixture is incubated at 37°C for 20 min,
35   followed by 93°C for 5 min. 50 cycles of PCR follow with each
cycles consisting of: denaturation for 1 min at 93°C; annealing
for 1 min at 55°C; and extension for 1.3 min at 72°C. After
the 50 cycles are complete, a further 10 min incubation at 72°C

follows. The reaction may be held at 4°C at this stage until
further analysis is undertaken.

To analyze the PCR amplified products, about one-
third to one-half of the PCR reaction volume is electrophoresed
5   on an agarose or polyacrylamide gel. DNA bands are visualized
by ethidium bromide staining, or, if additional sensitivity is
required, by Southern blotting of the PCR products followed by
probing with a labeled probe substantially complementary to the
transgene.
10

6. Early Detection of Transgenesis by ISH in Bovine Embryos

Transgenesis can be detected by performing in situ
hybridization (ISH) on cells biopsied from bovine embryos,
generally at the morula or blastocyst stage. For example,
15   pronuclear injection of bovine embryos is used to introduce a
transgene, and the microinjected embryo is cultured and
develops to a morula or blastocyst stage, generally to at least
the eight-cell stage and preferably to the sixteen-cell stage.
A biopsy of cells from the cultured embryo is taken, usually
20   the biopsy sample consists of about two or three cells of the
embryo. The detection of one or more integrated transgene(s)
in the biopsy sample is informative for the biopsied embryo,
which can be propagated further (e.g., for producing cloned
hemi-embryos and/or for implantation). Two or three cell
25   biopsy of 16 cell-atage morulae generally result in about 70 to
90 percent survival rates of the biopsied morulae.

Transgenesis can also be detected by performing in
situ hybridization on cloned cleavage bovine embryos (i.e.,
30   embryos which have been cleaved and cloned subsequent to
pronuclear transgene microinjection). A cloned embryo is
synchronized in metaphase (e.g., by incubation in a medium
containing colcemid). An entire cloned cleavage embryo, or a
biopsy thereof, that is substantially synchronized in metaphase
35   (at least about 50 percent of the cells are in metaphase) can
be fixed and used for ISH analysis. The detection of one or
more integrated transgene(s) in the metaphase-synchronized
embryo clone is informative for the remaining clone(s) which

were not subjected to the metaphase-synchronization ISH
procedures. Metaphase synchronization can also be performed on
biopsy material, wherein the biopsy cells are incubated in a
suitable culture medium containing a mitotic arrest agent such

5   as colcemid.

The metaphase stage is typically preferred because
the integrated transgene DNA is easily discriminated from non-
integrated DNA because it is represented by hybridization
signals at the same location on both sister chromatids.

10   However, metaphase-synchronization of biopsied blastomeres
requires additional time and effort and the ISH procedure also
can be practiced on interphase cells, which are more easily
obtained. The criterion on which a cell, such as a biopsied
embryo cell, is judged to be transgenic on the basis of ISH is

15   that essentially the majority, usually all, of the biopsied
cells show the same general hybridization pattern, i.e., one or
two (depending on the stage of the cell cycle, G1 or G2)
distinct hybridization signals which are distinguishable over a
less intense diffuse background (if present). Unintegrated DNA

20   gives rise to a random staining pattern which is indicated by a
preponderance of the biopsy cells showing an indistinct
hybridization pattern.

Embryo Biopsy Procedure

25   Transgene DNA is introduced into bovine embryos,
typically by microinjection of pronuclei, and the resultant
putative transgenic embryos are cultured under suitable
culturing conditions. A biopsy of the embryo is taken, usually
at the 16-cell stage (morula) or from compacted embryos (later

30   than the 16-cell stage), and the biopsy comprises at least one
cell, typically at least two cells, optionally at least three
cells, sometimes more than three cells but always less than the
number of cells comprising the embryo, typically less than
about half the cells comprising the embryo, and most usually

35   less than about 25 percent of the cells comprising the embryo.
The biopsy is generally performed by forming a slit or
perforation in the zona pellucida (supra) and extracting the
cell(s) composing the biopsy sample from the embryo. The

biopsy cell(s) can then be cultured for metaphase
synchronization and spreading, or can be used directly for _in
situ_ hybridization essentially regardless of the stage of the
cell cycle, but usually are interphase cell(s).

5   When the biopsy cells are to be used for ISH directly
without metaphase synchronization, the embryo(s) are incubated
for about 15 to 30 minutes in medium depleted in calcium and
magnesium (e.g., $Ca^{+2}-Mg^{+2}$-free medium) prior to manipulation.
The biopsy cells are removed as described, and biopsy cell(s)
10   are then washed in TL-Hepes and placed directly on a microscope
slide with a minimum volume of TL-Hepes. The TL-Hepes is
carefully aspirated away, leaving the biopsy essentially free
of TL-Hepes. A drop of approximately 5μl of fixative is added
to the biopsy immediately and the cells are preferably spread
15   before the fixative is completely evaporated. Approximately
1μl of softening solution is added at about room temperature
(i.e., about 15 to 30° C, usually 20 to 25° C) and additional
softening solution is typically added continuously until the
biopsy is well-spread and clean. Once spread, prehybridization
20   and subsequent procedures are essentially as described
hereinbelow.

                         Metaphase Spread Procedure
            Embryo cells, either from whole embryos, cloned
cleaved embryos, or biopsies, may optionally be cultured in a
25   suitable medium containing a cell cycle blocking agent, such as
colcemid, to produce metaphase-synchronized cells. The
resultant metaphase-synchronized cells can then be analyzed for
transgenesis by ISH.

            By the following procedure approximately 80% or more
30   of the cells are synchronized in metaphase and suitable for
analysis by _in situ_ hybridization. Although described for
whole embryos, the procedure is generally applicable to
cultured biopsy cells and to cleavage embryos which develop
from a cultured biopsy sample.

35

Chromosome Preparation from Preimplantation Bovine Embryos
            (a) Synchronization. Cleavage bovine embryos are
collected at 48 hours after _in vitro_ fertilization and cultured

for approximately 6 hours in the presence of 0.1 μg/ml colcemid
(Sigma) by standard methods, e.g., 39°C in a gas phase of 5%
CO_2 in air in drops of M199 medium (0.1% fetal calf serum,
0.01% antibiotic/antimycotic solution) under oil.

5   (b) <u>Hypotonic treatment</u>. The embryos are transferred to
a watch glass containing 1.5 ml of precooled (4°C) 0.075 M KCl
and kept at 4°C for several minutes, usually not more than
about 30 minutes.

     (c) <u>Fixation</u>. Fixative (3:1 methanol/glacial acetic
10   acid) is freshly prepared and kept at -20°C until needed.
Three ml of precooled fixative is placed in a precooled thick
watch glass and the embryos are added. It is important that
the watch glass is thick enough to maintain the temperature of
the fixative reasonably constant during ensuing operation under
15   the microscope. If the temperature rises quickly, two problems
arise: (1) most of the embryos will burst, (2) the embryos do
not remain sunken at the bottom of the watch glass but float
around in the fixative, making it impossible to find and
collect them under the microscope. The fixation time is
20   approximately 5 minutes at -20°C in cases where the zona
pellucida is not intact (e.g., has been perforated with a glass
needle), but may be longer if the ZP is intact, usually not
more than about 20 to 30 minutes.

     (d) <u>Spreading</u>. Each embryo is removed from the watch
25   glass and placed with not more than 1 μl fixative on a grease-
free slide (preringed on the back with a diamond pencil) under
a stereo-microscope. Before the fixative completely
evaporates, not more than one μl of softening solution (equal
parts of methanol and 75% acetic acid, precooled to 4°C) is
30   added at room temperature and carefully observed to monitor the
degree of spreading. Application of the softening solution is
continued until the spread is satisfactory.

                <u>Pre-Hybridization Treatment of Slides</u>

35   Before the initiation of hybridization procedure an
individual metaphase spread on each slide is located by phase-
contrast microscopy and recorded for speedy detection of the
metaphase spread after the <u>in situ</u> hybridization procedure. On

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biopsy samples or cleavage embryos which are not metaphase-synchronized, speedy microscopic detection is achieved by simply drawing a circle, usually with an indelible felttip marker, on the backside of the microscope slide(s) at sample positions after embedding (infra).

5

   (a)   RNAse treatment. Each slide is incubated with approximately 100 μl RNAse (0.1 mg/ml) under a coverslip for about 60 minutes at 37°C in a slide-jar. At the end of incubation, the coverslip is removed by the addition of 100 ml of 2xSSC (Sambrook et al., 1989) and gentle shaking of the slide-jar for 5 minutes on the top of an automatic shaker. The slides are further washed three times with 2xSSC at room temperature. Throughout the entire procedure, all the washings require typically 5 minutes incubation at room temperature with constant gentle shaking unless specified otherwise. Slides are dehydrated by sequential washing with 70%, 90%, and 100% ethanol.

10

15

   (b)   Pepsin treatment. The slides are incubated in 100 ml of pepsin (0.1 mg/ml 0.01 M HCl) in a slide-jar for 10 minutes at 37°C, followed by two washings with PBS.

20

   (c) $MgCl_2$ treatment and fixation. The slides are incubated for 5 minutes with 50 mM $MgCl_2$/PBS in a slide-jar at room temperature, followed by incubation for 10 min with 1.0% formaldehyde/50 mM $MgCl_2$/PBS at room temperature. The slides are washed once with PBS and dehydrated by washing with 70%, 90%, and 100% ethanol.

25

Denaturation, Probe Preparation, and Hybridization.

30

   (a)   Denaturation. Chromosomes are denatured by incubation of the slides for 3 minutes at 80°C after application of 100 μl of denaturation solution (a mixture of 70 μl of 100 formamide, 10 μl of 20xSSC and 10 μl of 0.5 M phosphate, pH 7.0 and 10 μl of H_2O) per each slide and covered with coverslips. AFter denaturation, 100 ml of chilled (-20°C) 70% ethanol was poured into the slide-jar and the slides are dehydrated and air-dried at room temperature.

35

(b) _Probe Preparation_. One μg of DNA for the probe
is digested with DNase (0.001 ng/μl) overnight at 370°C, then
nick-translated in the presence of 40 μM biotin-11-dUTP (Sigma)
for 1 hour at 14°C. The reaction is stopped by addition of 0.5

5   M EDTA. The labelled prove is purified by running on a G-50
sephadex column. The degree of labelling of the probe is
monitored by using BluGENE (BRL), a nonradioactive nucleic acid
detection system. Probe (20 ng per slide) and sheared bovine
genomic DNA (10 μg per slide) is dissolved in a desired volume

10   of hybridization solution (a mixture of 2.5 μl 100% formamide,
0.5 μl 20xSSC, 0.5 μl 0.5 M phosphate (pH 7.0), 1.5 μl H_2O and
5 μl 20% dextran sulfate. The probe solution is then denatured
by heating at 75°C for 5 minutes followed by quick chilling to
4°C. The probe is further incubated for at least 2 hours at

15   37°C to block out repetitive sequences before application to
slides (10 μl per slide).

         (c) _Hybridization_. Coverslips are boiled for 20
minutes in 0.2 M HCl and washed four times each with tap water,
deionized water, distilled water, and 100% ethanol. The

20   coverslips are dried and baked for at least 2 hours at 180°C.
The slides are covered with coverslips after application of 10
μl of probe, sealed with glue (Simson), and incubated for
approximately 16 hours at 37°C.

25   _Post-Hybridization Washing_.
         The coverslips loosened by 100 ml of washing solution
A (50% formamide/2xSSC, adjusted to pH 7.0 with HCl and
prewarmed to 45°C). After coverslips are removed, the slides
are washed three times with solution A.

30   The slides are further washed three more times with
washing solution B (0.1xSSC, 60°C). As described previously,
each washing takes 5 minutes with shaking of the slide-jar. It
is important that the temperature is maintained either at 45°C
or 60°C throughout the washing procedure. One additional

35   washing is carried out with washing solution C (4xSSC/0.05%
Tween 20) at room temperature.

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Immunocytochememical amplification

(a) Blocking. Each slide is incubated with 100 μl
of blocking solution (4xSSC/5% non-fat dry milk) with a
coverslip for 10 minutes at room temperature. The coverslip is
5   removed by shaking the slide-jar with 100 ml of washing
solution C.

(b) Immunocytochemical Reactions. One hundred μl of
fluorescein avidin-D FITC (Vector Lab) (diluted to 5 μg/ml with
blocking solution, followed by spinning in a microfuge for 1.5
10 minutes to remove dust particles) is applied to each slide,
covered with a coverslip, and incubated for 20 minutes at room
temperature. The coverslip is removed by shaking the slide-jar
with 100 ml of washing solution C. Slides are washed two
additional times with washing solution C.

15   This incubation and washing procedures are repeated
with 100 μl of biotinylated goat-anti-avidin D (Vector Lab)
(diluted to 5 μg/ml with blocking solution and centrifuged) per
slide.

The third immunocytochemical reaction is carried out
20 with fluorescein avidin-D FITC solution as described above.

The slides are dehydrated by washing with 100 ml of
70%, 90%, and 100% ethanol.

Embedding

25   Embedding solution is made by dissolving 2 gm of 1,4-
Diazabicyclo-(2,2,2)-octane (DABCO) in 90 ml of glycerol for
15-30 minutes at 60°C and 10 ml of 1.0 M Tris-HCl (pH 7.5) is
added and the pH is adjusted to 8.0 with a few drops of 5 M
HCl. The solution is cooled to room temperature before the
30 addition of 100 μl of Thimerosal solution (20%) and 100 μl of
propidium iodide solution (1 mg/ml). 35 μl embedding solution
is applied to each slide and covered with a coverslip before
fluorescent-microscopic (Olympus) examination.

35 ### Early Detection of Transgenesis in Murine Embryos by In Situ Analysis

Two to three day old embryos from a mating between a
hLF transgenic male and a non-transgenic female were

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synchronized with colcemid for 4 hours. About 15% of the cells
(usually ±3 out of 20) are arrested in metaphase. In situ
hybridization on the metaphase spreads with a biotinylated hLF
cDNA construct (by the procedure described above) showed that

5   40-50% of the embryos are hLF positive as manifested by
visualization of two clear fluorescent spots at identical
locations in the sister chromatids. The positive
identification was possible in embryos derived from mating of a
transgenic male carrying only a single copy hLF cDNA transgene.

10   Next, embryos were analyzed which were derived from
non-transgenic parents but injected in the pronuclear stage
with hLF cDNA constructs. As described above, double
fluorescent spots could be detected in a number of embryos, but
in the presence of a highly fluorescent background. We believe

15   the background originated from injected DNA which is neither
incorporated in nor cleared from the nuclei. The combination
of the background fluorescence and the low number of metaphase
spreads per embryo does not allow unambiguous identification of
the transgenic embryos.

20   In the third set of experiments the number of
metaphase spreads per embryo was increased. Control embryos (3
days old) were synchronized in the S-phase using varying
concentrations of colchicine before being release to and
subsequent blocked in mitosis. Incubation for 16 hours in a

25   low concentration of colchicine (<10 ng/ml) resulted in the
highest mitotic index, i.e., 75% metaphase spreads per embryo
(15 out of 20 cells).

              While the invention has been described in connection
with certain embodiments thereof, it should be recognized that

30   various modifications as may be apparent to one of skill in the
art to which the invention pertains also fall within the scope
of the invention as defined by the appended claims. The scope
of the invention should, therefore, be determined not with
reference to the above description, but should instead be

35   determined with reference to the appended claims, along with
the full scope of equivalents to which such claims are
entitled.

<u>Validation of ISH Method by Comparison to PCR Method</u>

Experiments were performed to validate the results
obtained from performing ISH on biopsy material by analyzing
the remaining biopsied embryo with the PCR method and comparing
5   the results of the two methods. In general, the results
obtained by both methods agree with each other. The biopsy
method was also validated by comparing the results produced by
ISH of 2-3 cell biopsies with the results obtained by ISH of
the remaining cells in the biopsied embryo.
10

<u>Experiment 1</u>

This experiment compares the results obtained by ISH
of about one-half of a split embryo to results obtained from
PCR analysis of the remainder of the biopsied embryo.
15   Transgenic bovine embryos were produced by pronuclear
microinjection. The embryos were cultured and developed to
the morula/blastocyst stage, at which time the embryo was split
and one half analyzed by <u>in situ</u> hybridization (ISH) and the
other by PCR. The results are shown in Table 1: plus (+)
20   indicates a result that is positive for integrated transgene
DNA, minus (-) indicates a negative result indicating the
absence of integrated transgene DNA, plus/minus (+/-) indicates
an ambiguous result, and "mos." indicates that the embryo
sample was mosaic, with some cells positive and other cells
25   negative. "N.D." indicates the sample was not determined.

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Table 1

Embryo	ISH	PCR
1	+	+
2	−	−
3	−	?
4	−	−
5	+	+
6	+	−
7	−	−
8	mos.	−
9	mos.	−
10	+/−	+
11	−	−
12	?	−
13	−	−
14	N.D.	N.D.
15	+/−	−
16	−	−
17	−	−
18	−	?

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Experiment 2

Experiment 2 compares the results obtained by ISH of
2-3 cell biopsies of embryos at the morula stage to results
obtained from ISH analysis of the remainder of the biopsied
5   embryos which were subsequently expanded to the blastocyst
stage. Transgenic bovine embryos were produced by pronuclear
microinjection. The embryos were cultured and developed to the
morula stage, at which time the embryo was biopsied and the 2-3
cell biopsy samples analyzed by in situ hybridization (ISH) and
10 the remainder of the embryo was cultivated to the blastocyst
stage and analyzed by ISH on the entire blastocyst. The results
are shown in Table 2: plus (+) indicates a result that is
positive for integrated transgene DNA, minus (-) indicates a
negative result indicating the absence of integrated transgene
15 DNA, plus/minus (+/-) indicates an ambiguous result, and "mos."
indicates that the embryo sample was mosaic, with some cells
positive and other cells negative. The percentages indicate
the percentage of cells in the remainder embryo that had the
phenotype indicated by biopsy ISH. Thus, for example, in
20 Embryo 6, the biopsy scored positive for integrated transgene
DNA and 23 cells of the remainder embryo (which consisted of 33
cells) scored positive for integrated transgene DNA, which is
70 percent of the remainder embryo cells.

Table 2

Embryo		Biopsy	Remaining Embryo
5	1	−	− (100%)
	2	−	− (100%)
	3	−	− (100%)
	4	+	+ (74%, 17/23)
	5	+	+ (70%, 23/33)
10	6	+	+ (70%, 23/33)
	7	+	+ (87%, 23/32)
	8	+(?)	+ (73%, 8/11)
	9	+/− mos.	mos. (42% +)
	10	+/− mos.	mos. (58% +)
15	11	+/− mos	mos. (58% +)
	12	+/− mos.	mos. (55% +)
	13	+/− mos.	mos. (50% +)
	14	−	− (95% −)
	15	+/− mos.	mos. (49% +)
20	16	+/− mos.	mos. (58% +)
	17	−	− (78% −)
	18	−	− (97% −)
	19	+/− mos.	mos. (45% +)

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Experiment 3

Experiment 3 compares the results obtained by ISH of
2-3 cell biopsies of embryos at the morula stage to results
obtained from PCR analysis of the remainder of the biopsied
5   embryos which were subsequently expanded to the blastocyst
stage. Transgenic bovine embryos were produced by pronuclear
microinjection. The embryos were cultured and developed to the
morula stage, at which time the embryo was biopsied and the 2-3
cell biopsy samples analyzed by _in situ_ hybridization (ISH) and
10   the remainder of the embryo was cultivated to the blastocyst
stage and analyzed by PCR on the entire blastocyst.

On the basis of several embryos analyzed by this
procedure, the ISH and PCR results correlate, with the ISH
method identifying a few positive integrated transgenics that
15   were scored as negative by PCR.

Experiment 4

Transgenic murine embryos were produced by pronuclear
microinjection and developed to the eight-cell stage. Two-cell
20   biopsies were performed and analyzed by ISH. Embryos that were
scored as positive or negative for integrated transgene DNA (in
blastocyst stage) were transferred to pseudopregnant foster
mothers. Of 17 biopsies analyzed, there were 2 positives, 12
negatives, and 3 indeterminates (+/-) scored. DNA from the
25   resultant pups is analyzed by Southern blotting in order to
check the prediction by the biopsy ISH.

30

WHAT IS CLAIMED IS:

1.   A method for the detection of an integrated transgene in
the nuclear genome of an embryo of an animal, said method
5   comprising the steps of:
        a)   performing in situ hybridization on at least one
cell from said embryo with a nucleic acid probe substantially
complementary to a transgene sequence; and
        b)   detecting a discrete hybridization signal in a
10   nucleus of said cell.

2.   A method of claim 1 further comprising the step of
removing said cell from said embryo prior to said in situ
hybridization step, thereby producing a viable biopsied embryo.

15

3.   A method of claim 1 wherein said embryo is an 8 to 16 cell
embryo or a compacted embryo.

4.   A method of claim 1 wherein said cell is an interphase
20   cell.

5.   A method of claim 1 wherein said probe is at least about 2
kilobases.

25   6.   A method of claim 1 wherein said animal is a bovine
species or murine species.

7.   A method of claim 1 further comprising the steps of
determining the sex of said embryo by hybridizing a labeled
30   probe that specifically hybridizes to a sex-determining
sequence and determining whether said sex-determining sequence
is present in the genome of said cell.

8.   A method for the detection of an integrated transgene in
35   the nuclear genome of an embryo of an animal, said method
comprising the steps of:
        a)   removing at least one cell from said embryo,
thereby producing a viable biopsied embryo and a biopsy sample;

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        b)   performing _in situ_ hybridization on said at least one cell from said biopsy sample with a nucleic acid probe substantially complementary to said transgene; and

        c)   detecting a discrete hybridization signal in the
5   nucleus of said cell.

9.   A method of claim 8 further comprising the step of determining the sex of said embryo by hybridizing a labeled probe that specifically hybridizes to a sex-determining
10   sequence and determining whether said sex-determining sequence is present in the genome of said cell.

10.   A viable biopsied embryo produced according to any of claims 1-9.
15

11.   A method for the detection of an integrated transgene in the nuclear genome of an embryo of an animal, said method comprising the steps of:

        a)   cloning said embryo, thereby producing a
20   multiplicity of cloned embryos;

        b)   performing _in situ_ hybridization on at least one cell from said cloned embryo with a nucleic acid probe substantially complementary to said transgene; and

        c)   detecting a discrete·hybridization signal in the
25   nucleus of said cell.

12.   A method of claim 11 further comprising the step of removing at least one cell from said embryo, thereby producing a viable biopsied embryo.
30

13.   A method of claim 11 wherein said embryo is an 8 to 16 cell embryo.

14.   A method of claim 11 wherein said cell is an interphase
35   cell.

15.   A method of claim 11 wherein said step of cloning is by nuclear transfer.

16. A method of claim 11 wherein said probe is at least about two kilobases.

17. A method of claim 11 wherein said animal is a bovine
5   species or murine species.

18. A method for the detection of an integrated transgene in the nuclear genome of an 8 to 16 cell embryo of an animal, said method comprising the steps of:
10   a) cloning said embryo by nuclear transfer, thereby producing a multiplicity of cloned embryos;
            b) removing at least one cell from at least one said cloned embryos, thereby producing a viable biopsied embryo and a biopsy sample;
15   c) performing in situ hybridization on said at least one cell of the biopsy sample with a nucleic acid probe substantially complementary to said transgene; and
            d) detecting a discrete hybridization signal in the nucleus of said cell.
20

19. A method for the determination of the sex of an embryo, said method comprising the steps of:
            a) removing at least one cell from an embryo, thereby producing a viable biopsied embryo and a biopsy sample;
25   c) performing in situ hybridization on said at least one cell of the biopsy sample with a labeled probe substantially complementary to a sex-determining sequence; and
            d) detecting a discrete hybridization signal in a nucleus of said cell.
30

20. A method of claim 11, 18, or 19, wherein the cell is an interphase cell.

21. A viable biopsied embryo produced according to any of
35 claims 11, 18, 19, or 20.

22. A method for detecting integration of a transgene into the nuclear DNA of an embryo of an animal and replication of said

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transgene as part of said nuclear DNA, wherein said integration
and replication provides for a methylated restriction site of
said transgene becoming an unmethylated restriction site, said
method comprising the steps of:

5   a)   treating said nuclear DNA from at least one cell
from said embryo with a restriction enzyme capable of cleaving
said methylated restriction site and incapable of cleaving said
unmethylated restriction site, thereby producing restriction
fragments of said nuclear DNA;

10   b)   amplifying said restriction fragments by PCR with
PCR primers substantially complementary to sequences on
different strands of said transgene and flanking said
unmethylated restriction site, thereby forming an amplified
transgene; and

15   c)   detecting said amplified transgene.

23.   A method of claim 22 wherein said PCR primers comprise
sequences other than ATGAAACTTATCCTCACCTGTCTTGTG or
GGGTTTTCGAGGGTGCCCCCGAGGATGGAT.

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24.   A method of claim 22 further comprising the step of
removing at least one cell from said embryo, thereby producing
a viable biopsied embryo.

25   25.   A method of claim 22 wherein said restriction enzyme is
DpnI and said methylated restriction site is methylated at N6
of an adenine of said methylated restriction site.

26.   A method of claim 22 wherein said embryo is an 8 to 16
30   cell embryo.

27.   A method of claim 22 wherein said animal is a bovine
species.

35   28.   A method of claim 22 wherein said PCR primers comprise at
least about 16 nucleotides.

29.   A method of claim 22 further comprising the steps of:
        performing in situ hybridization on at least one cell
from said embryo with a nucleic acid probe substantially
complementary to said transgene; and

5   detecting a discrete hybridization signal in the
nucleus of said cell.

30.   A method of claims 22-29, further comprising the step of
determining the sex of said embryo by hybridizing a labeled

10   probe that specifically hybridizes to a sex-determining
sequence and determining whether said sex-determining sequence
is present in the genome of said cell.

31.   A method for detecting integration of a transgene into the

15   nuclear DNA of an 8 to 16 cell embryo of an animal and
replication of said transgene as part of said nuclear DNA,
wherein said integration and replication provides for a
methylated restriction site of said transgene becoming an
unmethylated restriction site, said method comprising the steps

20   of:
        a)   removing at least one cell from said embryo,
thereby producing a viable biopsied embryo;
        b)   treating said nuclear DNA from said removed cell
with a restriction enzyme capable of cleaving said methylated

25   restriction site and incapable of cleaving said unmethylated
restriction site, thereby producing restriction fragments of
said nuclear DNA;
        c)   amplifying said restriction fragments by PCR with
PCR primers substantially complementary to sequences on

30   different strands of said transgene and flanking said
unmethylated restriction site, thereby forming an amplified
transgene; and
        d)   detecting said amplified transgene.

35   32.   A viable biopsied embryo produced according to any of
claims 22-31.

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33.   A method for detecting a transgene in an embryo of an
animal, said method comprising the steps of:
      a)   cloning said embryo, thereby producing a
multiplicity of cloned embryos;
5   b)   amplifying DNA from at least one cell of at least
one of said cloned embryos by PCR, thereby forming an amplified
transgene; and
      c)   detecting said amplified transgene.

10   34.   A method of claim 33 wherein:
      said transgene is integrated into the nuclear genome
of said animal and replicated as part of said genome, and said
integration and replication provides for a methylated
restriction site of said transgene becoming an unmethylated
15 restriction site;
      said step of performing PCR employs PCR primers
substantially complementary to sequences on different strands
of said transgene and flanking said unmethylated restriction
site; and
20   said method further comprises the step of treating
said nuclear DNA from at least one cell of at least one of said
cloned embryos with a restriction enzyme capable of cleaving
said methylated restriction site and incapable of cleaving said
unmethylated restriction site.
25

35.   A method of claim 33 wherein said restriction enzyme is
Dpn I and said methylated restriction site is methylated at N6
of an adenine of said methylated restriction site.

30 36.   A method of claim 33 further comprising the step of
removing at least one cell from at least one of said cloned
embryos, thereby producing a viable biopsied embryo.

37.   A method of claim 33 wherein said step of amplifying DNA
35 further comprises the steps of:
      cleaving said DNA with a restriction enzyme incapable
of cutting within said transgene, thereby producing restriction
fragments of said DNA;

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adding ligase to said restriction fragments under conditions which result in self ligation of said restriction fragments, thereby producing circularized restriction fragments;

5   amplifying said circularized restriction fragments by inverse PCR, thereby producing an amplified transgene; and detecting said amplified transgene.

38.   A method of claim 33 wherein said step of cloning is by
10   nuclear transfer.

39.   A method of claim 33 wherein said embryo is an 8 to 16 cell embryo.

15   40.   A method of claim 33 wherein said animal is a bovine species.

41.   A method of claim 40, wherein said animal is a cow.

20   42.   A method of claim 33 wherein said PCR primers comprise about 16 nucleotides.

43.   A method according to claims 33-41, further comprising the step of determining the sex of the embryo by hybridizing a
25   labeled probe that specifically hybridizes to a sex-determining sequence and determining whether said sex-determining sequence is present in the genome of said cell.

44.   A method for detecting integration of a transgene into the
30   nuclear DNA of an 8 to 16 cell embryo of an animal and replication of said transgene as part of said nuclear DNA, wherein said integration and replication provides for a methylated restriction site of said transgene becoming an unmethylated restriction site, said method comprising the steps
35   of:
           a)   cloning said embryo by nuclear transfer, thereby producing a multiplicity of cloned embryos;

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       b)   removing at least one cell from said cloned
embryos, thereby producing at least one viable biopsied embryo;

       c)   treating said nuclear DNA from said removed cell
with a restriction enzyme capable of cleaving said methylated

5   restriction site and incapable of cleaving said unmethylated
restriction site, thereby producing restriction fragments of
said nuclear DNA;

       d)   amplifying said restriction fragments by PCR with
PCR primers substantially complementary to sequences on

10   different strands of said transgene and flanking said
unmethylated restriction site, thereby forming an amplified
transgene; and

       e)   detecting said amplified transgene.

15   45.   A method for detecting integration of a transgene into the
nuclear DNA of an 8 to 16 cell embryo of a bovine species and
replication of said transgene as part of said nuclear DNA,
wherein said integration and replication provides for a
methylated Dpn I restriction site of said transgene becoming an

20   unmethylated Dpn I restriction site, said method comprising the
steps of:

       a)   cloning said embryo by nuclear transfer, thereby
producing a multiplicity of cloned embryos;

       b)   removing at least one cell from said cloned

25   embryos, thereby producing at least one viable biopsied embryo;

       c)   treating said nuclear DNA from said removed cell
with Dpn I, thereby producing Dpn I restriction fragments of
said nuclear DNA;

       d)   amplifying said Dpn I restriction fragments by

30   PCR with PCR primers substantially complementary to sequences
on different strands of said transgene and flanking said Dpn I
restriction site, thereby forming an amplified transgene; and

       e)   detecting said amplified transgene.

35   46.   A viable biopsied embryo produced according to any of
claims 33-44.

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47. A method for detecting integration of a transgene into the
nuclear DNA of an embryo of an animal and replication of said
transgene as part of said nuclear DNA, wherein said integration
and replication provides for a methylated restriction site of
5   said transgene becoming an unmethylated restriction site, said
method comprising the steps of:
         a)   treating said nuclear DNA from at least one cell
from said embryo with a restriction enzyme capable of cleaving
said methylated restriction site and incapable of cleaving said
10 unmethylated restriction site, thereby producing restriction
fragments of said nuclear DNA;
         b)   amplifying said restriction fragments by PCR with
PCR primers substantially complementary to sequences on
different strands of said transgene and flanking said
15 unmethylated restriction site, thereby forming an amplified
transgene;
         c)   detecting said amplified transgene;
         d)   performing _in situ_ hybridization on at least one
metaphase stage cell from said embryo with a nucleic acid probe
20 substantially complementary to said transgene; and
         e)   detecting a hybridization signal on both sister
chromatids of a chromosome of said cell.

48. A method for detecting a transgene in an embryo of an
25 animal, said method comprising the steps of:
         a)   cloning said embryo, thereby producing a
multiplicity of cloned embryos;
         b)   amplifying DNA from at least one cell of said
cloned embryos by PCR, thereby forming an amplified transgene;
30   c)   detecting said amplified transgene;
         d)   performing _in situ_ hybridization at least one
cell from said cloned embryos with a nucleic acid probe
substantially complementary to said transgene; and
         e)   detecting a hybridization signal in a nucleus of
35 said cell.

49. A method according to claims 44-48, further comprising the
step of determining the sex of the embryo.

50. A viable embryo produced according to any of claims 44-49.

INTERNATIONAL SEARCH REPORT

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12N 15/00; C12Q 1/68
US CL   :435/6, 172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Developmental Biology, Volume 101, issued 1984, K. H. Cox et al, "Detection of mRNAs in Sea Urchin Embryos by in situ Hybridization using Asymmetric RNA Probes", pages 485-502, entire document.	1-9,11-20,20,30,43 and 47-49
X	R. B. Church, "Transgenic Models in Medicine and Agriculture" published 1990 by Wiley-Liss, Inc., pages 33-45.	10 and 21-50

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 August 1992	27 AUG 1992
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